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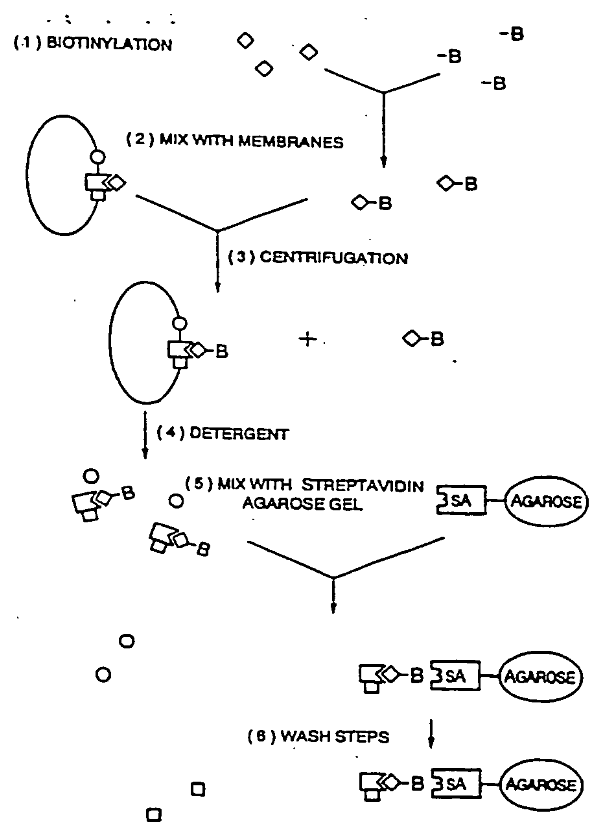
(54) Title: A METHOD FOR ISOLATING AND PURIFYING TRANSFERRIN AND LACTOFERRIN RECEPTOR PROTEINS FROM BACTERIA AND THE PREPARATION OF VACCINES CONTAINING THE SAME

(57) Abstract

This invention provides methods for isolating and purifying transferrin and lactoferrin receptor proteins from bacterial pathogens by affinity chromatography and provides the preparation of vaccine antigens comprising the purified transferrin and iactoferrin receptor proteins.



A METHOD FOR ISOLATING AND PURIFYING TRANSFERRIN AND LACTOFERRIN RECEPTOR PROTEINS FROM BACTERIA AND THE PREPARATION OF VACCINES CONTAINING THE SAME

Background Of The Invention

The present invention relates to a method for isolating and purifying transferrin and lactoferrin receptor proteins from bacterial pathogens and to vaccines containing purified transferrin and/or lactoferrin receptor proteins and/or their derivatives.

There are a number of important bacterial pathogens causing disease in humans and in animals for which effective vaccines are either absent or unsatisfactory. A number of these pathogens are relatively host specific with respect to their ability to cause natural infection. Bacteria such as Neisseria meningitidis, Haemophilus influenzae and Neisseria gonorrhoeae continue to be an important cause of endemic and epidemic human diseases such as meningitis, otitis, epiglottitis, gonorrhea and urethritis. Similarly, Pasteurella haemolytica, Haemophilus somnus and Pasteurella multocida are important causative agents of pneumonic pasteurellosis and infectious thromboembolic meningoencephalitis in cattle. In pigs, Actinobacillus (Haemophilus) pleuropneumoniae is an important causative agent of infectious pneumonia. In poultry, the avian Haemophili, particularly Haemophilus paragallinarum, are responsible for infectious coryza.

Haemophilus influenzae and Neisseria meningitidis are the most common cause of bacterial meningitis in young children. Despite available effective antibiotic therapy, significant mortality and morbidity result from meningococcal infection. The fulminant nature of the infection, coupled with the scant characteristic

haemolytica have been inconsistent in reducing the incidence and severity of the disease. Infectious thromboembolic meningoencephalitis, an important cause of mortality in feedlot cattle, is caused by Haemophilus somnus. There is currently no effective vaccine for the prevention of this disease. Actinobacillus (Haemophilus) pleuropneumonia causes a contagious pneumonia in pigs which constitutes a major problem for the swine industry throughout the world. Vaccination with crude vaccine preparations have not been successful due to limited protection of heterologous serotypes. Infectious coryza in poultry, which is primarily caused by Haemophilus paragallinarum, results in significant reduction in productivity in the poultry industry.

Iron acquisition is essential for the growth and survival of bacterial pathogens in the host and for causing infection. Bacterial pathogens in the mammalian host are confronted with an environment in which the level of iron is extremely low. In the extracellular compartment, iron is sequestered by the proteins transferrin and lactoferrin, which predominate in serum and mucosal secretions, respectively. The ability to compete with lactoferrin and transferrin for iron is thought to be essential for the pathogenesis of many bacterial infections. Many bacteria manufacture iron-chelating compounds known as siderophores to facilitate iron acquisition from their environment. However, several pathogenic bacteria, such as Neisseria meningitidis, Neisseria gonorrhoeae, and Haemophilus influenzae do not produce siderophores, but rather acquire lactoferrin iron and transferrin iron directly for growth in vitro.

Early observations of meningococci and gonococci by B.E. Holbein, I.W. DeVoe and F.P. Sparling and co-workers demonstrated that these bacteria can grow in

proteins previously been isolated and purified. Further, no vaccine containing the receptor proteins has been previously developed.

Summary Of The Invention

The present invention overcomes the problems and disadvantages of the prior art by providing a method for isolating and purifying the lactoferrin and transferrin receptor proteins thereby facilitating the production of vaccines containing the lactoferrin and/or transferrin receptor proteins.

It is an object of the invention to provide a method for identifying lactoferrin and transferrin receptor proteins in bacterial pathogens and isolating and purifying the same.

It is also an object of the invention to provide single component vaccine antigens that are effective in the prevention of diseases caused by bacterial pathogens containing lactoferrin and transferrin receptor proteins.

It is a further object of the invention to provide vaccine antigens that are effective in preventing bacterial pathogen diseases in young children.

It is an additional object of the invention to provide vaccine antigens that exhibit superior immunological memory to current polysaccharide capsular vaccines.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the instrumentalities and combinations, particularly pointed out in the appended claims.

the amino acid sequence of a purified transferrin receptor protein or on the nucleotide sequence of a cloned receptor gene. The preparation may be suspended in 0.15 M sodium chloride, 0.05 M sodium phosphate, a buffer having a pH of about 7.4, thimerosal and optionally, an adjuvant.

The single-component vaccine antigens of the invention are effective against bacterial pathogens that acquire iron directly through transferrin and/or lactoferrin receptors. The vaccine antigens are also suitable for providing immunity to young children.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates an embodiment of the invention, and together with the description, serves to explain the principles of the invention.

Brief Description Of The Drawings

The drawing is a flow chart of an affinity chromatography method according to the present invention.

Description Of The Preferred Embodiments

Reference will now be made in detail to the present preferred embodiments of the invention.

The lactoferrin receptor is a surface-accessible outer membrane protein. It has been found to have a molecular weight of about 106,000 in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The receptor is specific for binding lactoferrin. It does not bind transferrin or any other iron-binding proteins. Further, the receptor from *Neisseria* and *Branhamella* species binds human lactoferrin with high affinity but does not specifically bind lactoferrin from other species. Expression of the receptor is regulated by the level of iron available to the bacteria possessing

or turkey) transferrin, but not with transferrins for mammalian species, i.e., human, porcine or bovine.

The transferrin receptor consists of two outer membrane proteins: (1) a higher molecular weight protein of about 100,000 in *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Haemophilus influenzae*; and (2) a lower molecular weight protein of from about 65,000 to about 90,000 in various strains and species. In some species, such as *Neisseria meningitidis*, the lower molecular weight protein can partially reconstitute transferrin binding activity after sodium dodecyl sulfate polyacrylamide electrophoresis and electroblotting. Both purified receptor proteins from *Neisseria meningitidis* can partially reconstitute transferrin binding activity after elution from an affinity column with guanidine hydrochloride and removal of the guanidine hydrochloride.

The transferrin receptor binds transferrin but not other iron-binding proteins. In the human pathogens *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Branhamella catarrhalis* and *Haemophilus influenzae*, the receptors bind human transferrin with high affinity but do not specifically bind transferrin from other species. In the bovine pathogens *Pasteurella haemolytica*, *Haemophilus somnus*, and *Pasteurella multocida*, the receptors bind bovine transferrin but not transferrins from other species. In the pig pathogens *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, and *Haemophilus suis*, the receptors bind porcine transferrin but not transferrins from other species. In the poultry pathogens *Haemophilus paragallinarum* (*Haemophilus gallinarum*), and *Haemophilus avium*, the receptors bind avian (chicken or turkey) transferrins, but not transferrins from mammalian species. The transferrin receptor from human pathogens binds iron-saturated human transferrin

the resin, the bound proteins were eluted by boiling in sample buffer and analyzed by SDS-PAGE. Alternatively, the lactoferrin receptor protein can be eluted by increasing concentrations of guanidine hydrochloride.

A protein having a molecular weight of about 105,000 was bound to the lactoferrin affinity resin when total membrane from iron-starved N. meningitidis B16B6, Group X and Group W135 was used.

The mechanism of iron acquisition from transferrin in meningococci involves binding by a receptor on the surface of the bacterium, and the lack of accumulation of ^{125}I-transferrin indicates that uptake is not due to internalization of a transferrin-receptor complex. It is believed that the removal of iron from transferrin and the release of apotransferrin are subsequent steps in the uptake mechanism. There is a higher affinity of the transferrin receptor for iron-saturated transferrin than for apotransferrin. Since iron acquisition from lactoferrin also involves a surface receptor, it is believed that a similar mechanism of uptake exists for lactoferrin receptors.

Transferrin-binding activity was detected in all strains of Neisseria meningitidis, Haemophilus influenzae, N. gonorrhoeae, N. lactamica and B. catarrhalis tested. Transferrin binding activity in all isolates tested was specific for human transferrin in that only human transferrin could effectively block binding of horseradish peroxidase-conjugated human transferrin.

The transferrin receptor was previously identified by SDS-PAGE and Western blot analysis as a protein having a molecular weight of from about 75,000 to about 88,000. However, a pure transferrin receptor was not isolated by that procedure. Also, the higher molecular weight transferrin binding protein was not identified by that procedure. The inventor developed an affinity

The lactoferrin and/or transferrin receptor protein isolates are included in the vaccine antigens of the invention in a pharmaceutically effective amount to achieve sufficient immunogenicity.

The vaccine antigens of the invention can be administered by an effective route of administration well known to those of ordinary skill in the art, for example, sub-cutaneously or intramuscularly.

The invention will be further clarified by the following examples which are intended to be purely exemplary of the invention.

Example 1: Identification And Characterization Of The Human-Lactoferrin Binding Protein From Neisseria Meningitidis

Bacterial strains and growth conditions

N. meningitidis B16B6, a standard serotyping strain was obtained from C. Frasch. Group X and group W135 meningococcal strains were obtained from Foothills Hospital, Calgary, Alberta. Meningococci were grown on chocolate agar plates supplemented with CVA enrichment (GIBCO Laboratories, Grand Island, N.Y.) in an atmosphere containing 5% CO_2. Freshly grown cells from chocolate plates were routinely used to inoculate liquid Mueller-Hinton broth (MHB) to a starting A_{600} of 0.04 and were incubated with shaking for 16 hours prior to harvest. Iron starvation MHB normally contained 35 μM EDDA (ethylenediamine di-ortho-hydroxyphenylacetic acid). The broth and culture conditions used for expression experiments are indicated in Table 1.

Chemicals

Horseradish peroxidase-conjugated human lactoferrin was obtained from Jackson Immunoresearch Laboratories, Avondale, Pa. Bovine lactoferrin was from Accurate Chemicals, Westbury, N.Y. Human lactoferrin (L-8010),

NaCl and one change of 50 mM Tris hydrochloride, pH 7.5, concentrated by ultrafiltration with an Amicon Centriflo membrane cone and stored at 4°C.

Preparation of membranes

Cells were harvested and washed in 50 mM Tris hydrochloride, pH 7.5 buffer and resuspended at a concentration of 0.2 g of cells per ml in buffer containing 50 µg of phenylmethylsulfonyl fluoride per ml. After the cells were lysed by passage two times through a French pressure cell at 16,000 lb/in^2, cellular debris was removed by centrifugation at 8,000 x g for 15 min. Crude total membranes were collected by centrifugation at 140,000 x g for 1 hour and suspended in the above buffer. Outer membranes were prepared from crude total membranes by selective detergent extraction with Sarkosyl NL97. Total membranes were diluted to 5 mg of protein per ml. and Sarkosyl was added to 0.5%. The mixture was incubated on ice for 30 min. and the outer membranes were collected by centrifugation at 180,000 x g for 10 min. The pellet was resuspended in buffer and reextracted as above, and the final washed pellet was resuspended in buffer alone.

Batch affinity isolation of binding protein

20 µg of biotinylated human lactoferrin or transferrin was mixed with 0.75 mg of total membrane protein in 1 ml of 50 mM Tris hydrochloride 100 mM NaCl, pH 8.0 buffer and incubated with gentle agitation for 60 min. at 37°C. The membranes were pelleted by centrifugation at 16,000 x g for 10 min in an Eppendorf microcentrifuge and resuspended in 1 ml of buffer. EDTA was added to 10 mM and Sarkosyl was added to 0.75%, followed by 100 µl of a 1/2 dilution of streptavidin-agarose (Bethesda Research Laboratories,

1970). The SDS-PAGE gel was silver-stained according to the method of Oakley et al. (Anal. Biochem 105:361-363, 1980) with the following minor modifications. The gel was first fixed overnight with a solution of 25% isopropanol, 7% acetic acid. After removal of the developing solution, development was stopped with a solution of 0.35% acetic acid for 1 hour, and then the gel was washed with water.

Lactoferrin binding assay

The dot-binding assay for lactoferrin was performed essentially as described previously for transferrin-binding activity (A.B. Schryvers and L. Morris, Molecular Microbiology, 2:281-288, 1988) except that conjugated lactoferrin (250 to 500 ng/ml) was included in the binding mixture. The commercially prepared human lactoferrin has a ratio of peroxidase to lactoferrin of 1:1.5. Therefore, the concentration of conjugated lactoferrin used routinely ranged from approximately 1.8 to 3.6 nM (the average molecular weight of conjugated lactoferrin is 140,000). In competition experiments, mixtures of unconjugated proteins and conjugated human lactoferrin were prepared prior to application to the membrane.

For expression experiments requiring quantitation, cell suspensions were adjusted to an A_{600} of 10, and a series of nine two-fold dilutions were prepared and spotted onto the paper. In samples where significant binding protein expression was anticipated, the first dilution was a 10-fold dilution. A dilution series of the conjugated human lactoferrin was also applied directly to the same paper. After development with substrate and drying of the paper, the spots were measured with a BioRad model 620 Video Densitometer by using the reflectance setting and interfaced with a microcomputer with the Bio-Rad 1-D Analyst software

Table 1: Expression of Lactoferrin-Binding Activity

Addition(s) (μM) to growth medium[a]	Final A_{600}[b]	Bindingactivity[c] ng/mg	ng/ml
	a	a	a
None	3.8	<22	<43
EDDA (40)	1.8	5,800	3,300
EDDA (60)	1.4	7,000	4,100
EDDA (80)	1.1	7,300	3,400
EDDA (100)	0.9	7,600	2,700
+$FeCl_3$ (120)	3.6	<30	<50
+HHb (0.1)	1.7	7,600	5,200
+HHb (0.5)	2.3	7,800	6,400
+HHb (2.0)	2.6	280	330
+HHb (5.0)	3.7	<29	<43
+HHb (20)	3.8	<24	<44
+HTr (1.0)	1.8	7,100	4,100

a. Growth medium consisted of brain-heart infusion broth with the indicated additions, HHb. Human hemoglobin: HTr, iron-saturated human transferrin.

b. Cultures were inoculated with cells resuspended from fresh growth on chocolate plates to a starting A_{600} of 0.04 and incubated at 37°C for 16 h. The final A_{600} was measured after 16 h growth with medium containing the indicated additions as a blank.

c. Binding activity expressed as nanograms of conjugated lactoferrin bound per milligram of total cell protein or per milliliter of original culture volume was determined as described in the text.

RESULTS

Expression of lactoferrin-binding activity

To evaluate the regulation of expression of lactoferrin-binding activity in N. meningitidis, strain B16B6 was grown in broth containing a variety of

is consistent with previous observations that all meningococcal strains tested were capable of using lactoferrin iron for growth.

Identification of the lactoferrin binding protein

A batch method of affinity chromatography with biotinylated human lactoferrin and streptavidin-agarose was used to identify the lactoferrin-binding protein in several different meningococcal strains. A protein of approximately 105,000 molecular weight was specifically bound to the lactoferrin affinity resin when total membranes from iron-starved N. meningitidis B16B6 were used. When biotinylated lactoferrin was omitted from the procedure the band was absent, indicating that specific binding to lactoferrin was involved. The band was also absent when total membranes from iron-sufficient cells were used which is consistent with the observation that expression of lactoferrin-binding activity is strongly repressed by iron (Table 1). Although proteins of 70,000 and 38,000 molecular weight were also observed to copurify with the 105,000 molecular weight band when mild washing was performed, they were successively removed by more extensive washing procedures. Under the conditions of elution, very little biotinylated lactoferrin was released from the resin (80,000 molecular weight), but inclusion of a reducing reagent in the sample mix prior to boiling resulted in an increase in this band observed by SDS-PAGE. A minor band of 37,000 molecular weight was observed in virtually all samples. Affinity chromatography using total membranes from group X and group W135 meningococcal strains identified a lactoferrin-binding protein of a similar molecular weight. The band observed at 70,000 M_1 in these samples was due to inadequate washing, and the band at

prewarmed Brain Heart Infusion broth containing 100 μM EDTA to a starting A_{600} of 0.02. The resulting culture was incubated at 37°C with shaking for 16 hrs prior to harvest by centrifugation at 9,000 x g for 15 minutes. The cells were resuspended to 0.2 gm/ml in 50 mM TrisHCl, pH 8 buffer containing 50 μg/ml phenylmethylsulfonyl fluoride. The cells were lysed by passing the suspension through a French pressure cell at 16,000 lb/in^2 and cell debris was removed by centrifugation at 9,000 x g for 15 min. Crude total membranes were collected by centrifugation at 140,000 x g for 1 hr and resuspended in 50 mM TrisHCl, pH 8 buffer.

(b) Affinity Isolation of Receptor Proteins

0.9 mg of biotinylated human transferrin or biotinylated human lactoferrin prepared as described in Example 1 was mixed with 72 mg of crude total membrane protein in 60 mls of 50 mM TrisHCl, 100 mM NaCl, pH 8 buffer and incubated for 1 hr at 22°C. The mixture was centrifuged at 9,000 x g for 15 minutes to collect the membranes and the pellet was resuspended in 60 mls of the above buffer and incubated for 10 minutes at 22°C. EDTA was added to 10 mM and Sarkosyl was added to 0.75% and the mixture was incubated a further 10 minutes at room temperature prior to centrifugation at 9,000 x g for 15 min. The supernatant was mixed with 5 mls of Streptavidin-Agarose (1-2 mg streptavidin per ml resin) and incubated at room temperature with gentle mixing for 1 hr. The resin was collected by centrifugation at 500 x g for 10 minutes and resuspended in 80 mls of 50 mM TrisHCl, 1 M NaCl, 10 mM EDTA, 0.75% Sarkosyl, pH 8 buffer. The resin was again collected by centrifugation and washed two more times in the above buffer. After the final wash the resin was resuspended in 20 mls of the above buffer and poured into a 1 cm

twenty minutes, 20 mg of fully iron-loaded human transferrin in 1 ml of sterile saline was injected intraperitoneally into the same mice previously exposed to the challenge bacteria. The mice were observed for a total period of 72 hours and the number of dead and surviving mice were recorded.

TABLE 3

Group#	Immunizing Antigen* Primary	2nd/3rd/4th	exogenous hTf	#survivers/total**
1	none	none	yes	0/4
2	MDP	none	yes	0/4
3	MDP + receptor	receptor	yes	4/4
4	MDP + OM	OM	yes	4/4

*MDP - 50 μg of muramyl dipeptide, receptor - approximately 10 μg of transferrin receptor isolated from Neisseria meningitidis strain B16B6 as described in Example 2, OM - 50 μg of iron-deficient outer membranes isolated from Neisseria meningitidis by selective detergent extraction with Sarkosyl.
**Mice were challenged with 1 X 10^7 cells of meningococcal strain B16B6 grown on Mueller-Hinton agar plates containing 35 μM EDDA. Twenty minutes after injecting the challenge bacteria, 20 mg of fully iron-loaded human transferrin was injected intraperitoneally as a source of exogenous iron.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

6. The method of claim 5 wherein affinity chromatography is carried out using biotinylated human lactoferrin and streptavidin-agarose.

7. The method of claim 5 wherein said bacterial strain is selected from the group consisting of _Neisseria meningitidis_, _Neisseria gonorrhoeae_, _Neisseria lactamica_ and _Branhamella catarrhalis_ strains.

8. The lactoferrin receptor protein isolated and purified by the method of claim 5.

9. A vaccine antigen comprising a transferrin receptor protein preparation.

10. The vaccine antigen of claim 9 further comprising sodium chloride, sodium phosphate, buffer and thimersol.

11. The vaccine antigen of claim 9 wherein said transferrin receptor protein preparation is selected from the group consisting of a purified transferrin receptor protein isolated from an organism expressing at least one cloned transferrin receptor gene, a derivative of a purified transferrin receptor protein, a fusion protein having at least a portion of a coding sequence of at least one transferrin receptor gene, a synthetic peptide having an amino acid sequence based on the amino acid sequence of at least one purified transferrin protein, and a synthetic peptide having an amino acid sequence based on the nucleotide sequence of a cloned transferrin receptor gene.

12. The vaccine antigen of claim 9 further comprising an adjuvant.

13. The vaccine antigen of claim 12 wherein said adjuvant is aluminum hydroxide.

14. The vaccine antigen of claim 11 wherein said preparation is prepared from a strain selected from the group consisting of _Neisseria meningitidis_, _Haemophilus influenzae_, _Neisseria gonorrhoeae_,

20. The vaccine antigen of claim 16 further comprising an adjuvant.

21. The vaccine antigen of claim 20 herein said adjuvant is aluminum hydroxide.

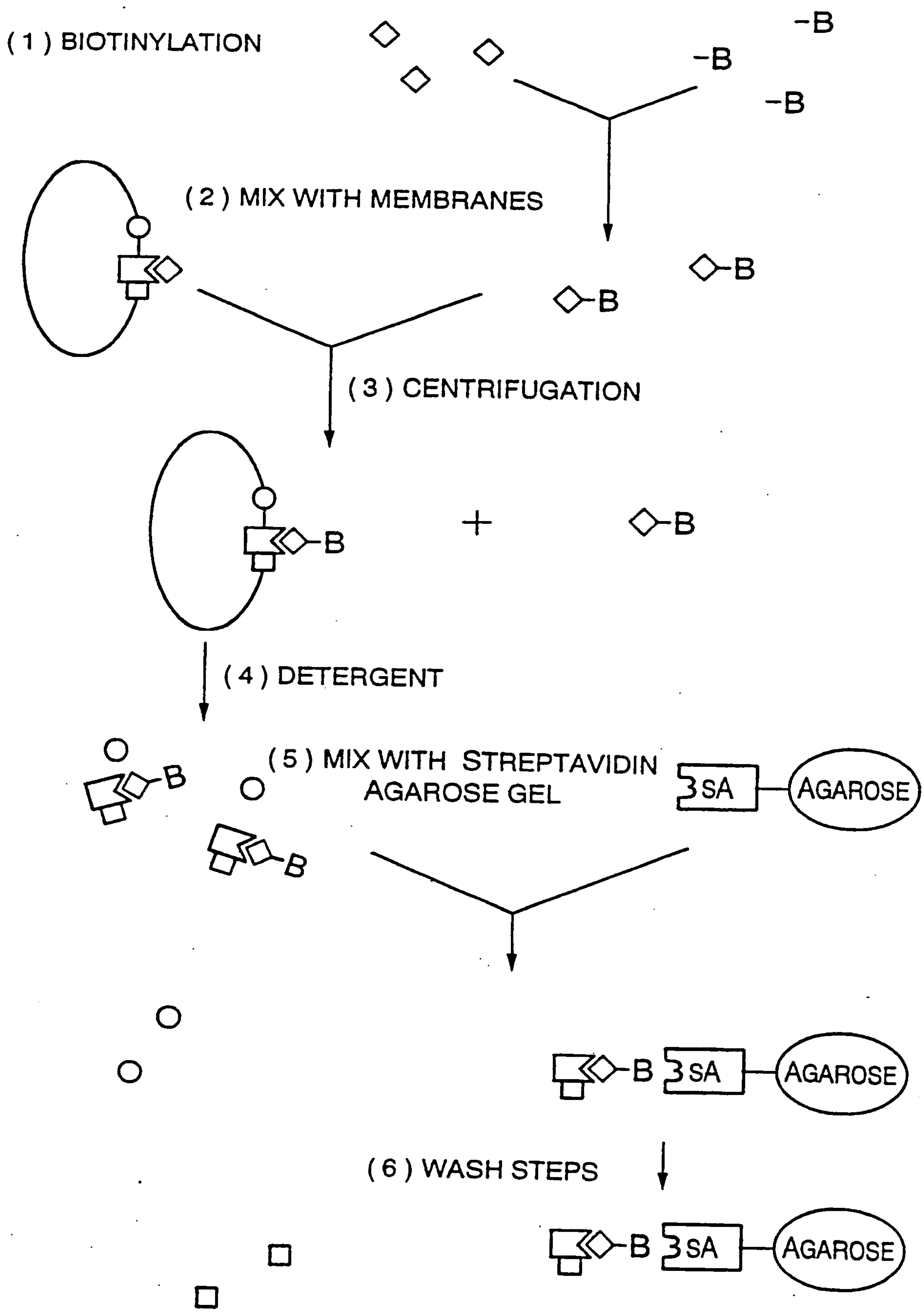
22. A vaccine antigen comprising a lactoferrin receptor protein preparation and a transferrin receptor protein preparation.

23. The vaccine antigen of claim 22 further comprising sodium chloride, sodium phosphate, buffer and thimersol.

24. The vaccine antigen of claim 22 further comprising an adjuvant.

25. The vaccine antigen of claim 24 wherein said adjuvant is aluminum hydroxide.

26. The vaccine antigen of claim 22 wherein said lactoferrin receptor protein preparation and said transferrin receptor protein preparation are prepared from at least one strain selected from the group consisting of <u>Neisseria meningitidis</u>, <u>Neisseria gonorrhoeae</u>, <u>Neisseria lactamica</u>, <u>Branhamella catarrhalis</u>, <u>Haemophilus influenzae</u>, <u>Pasteurella haemolytica</u>, <u>Pasteurella multocida</u>, <u>Haemophilus somnus</u>, <u>Actinobacillus pleuropneumoniae</u>, <u>Actinobacillus suis</u>, <u>Haemophilus suis</u>, <u>Haemophilus paragallinarum</u>, <u>Haemohilus gallinarum</u>, and <u>Haemophilus avium</u> strains.



(1) BIOTINYLATION
-B
-B
-B
(2) MIX WITH MEMBRANES
B
B
(3) CENTRIFUGATION
B
+
B
(4) DETERGENT
B
(5) MIX WITH STREPTAVIDIN
AGAROSE GEL
SA
AGAROSE
B
B
SA
AGAROSE
(6) WASH STEPS
B
SA
AGAROSE

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00131

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 39/095, A 61 K 39/102, A 61 K 39/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	J. Med. Microbiol., vol. 29, no. 2, 1989, The Pathological Society of Great Britian and Ireland, A.B. Schryvers: "Identification of the transferrin- and lactoferrin-binding proteins in Haemophilus influenzae", pages 121-130, see the whole article --	1-26
X	Can. J. Microbiology, vol. 35, no. 3, 1989, A. B. Schryvers et al.: "Comparative analysis of the transferrin and lactoferrin binding proteins in the family Neisseriaceae", pages 409-415, see the whole article --	1-8

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th July 1990	09. 08. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. SOTELO